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Re: Patent Application

Serial Number **10/031,874** of Tanha *et. al.*, Filed November 14, 2002;

Examiner David J. Blanchard

Declaration of Jamshid Tanha

I, Jamshid Tanha, am employed as research scientist at the National Research Council of Canada. I have been employed in this capacity since April 1997.

Except where otherwise noted, I have personal knowledge of the statements made herein.

I am a named inventor in respect of United States Patent Application Serial Number 10/031,874.

I have 13 years of experience in the area of recombinant antibody and phage display technologies and consider myself to be an expert in this field.

In my view, the use of a phage vector library, as opposed to the use of a phagemid vector, is a practical necessity for the production of a phage display library suitable for production and generation of useful VHH binders from a non-immunized camelid source.

My belief is based on my years of experience in this field, and my reading and research in the area.

In addition to other related work, I compared the effectiveness of phage vectors and phagemid vectors for the production of phage display libraries for VHH single domain antibodies. A description of this work appears below. All the work was conducted by me personally, or by competent staff under my immediate supervision. I fully believe that the results reported below are typical for this type of comparative study. In my opinion, these results prove that phagemid vectors are wholly unsuitable for use in the generation of VHH phage display libraries from a non-immunized camelid source.

DESCRIPTION OF WORK:

Rescuing Binders from Naïve VHH Phage Display Libraries Requires a Phage Vector-based Approach

1. ABSTRACT

The display of recombinant antibody libraries on the surface of the filamentous bacteriophages is generally achieved by employing a phagemid vector/helper phage system or a phage vector. In the former approach, more than 90% of the phages in the phage display library do not display recombinant fragments and the remaining that do, essentially display one copy. In the latter approach, essentially every phage in the library displays 3-5 copies of the recombinant antibody fragments. This results in a very high "effective" concentration of binders in a phage-vector-based library compared to a phagemid-vector one during the selection steps when the binders come in contact with the target antigen. Thus, it is conceivable that in cases where the affinity of the binders in a library is low, as is the case with naïve VHH libraries, the vector type would determine the success of isolating binders. To explore the effect of vector type, i.e., phagemid vector versus phage vector, in enabling naïve VHH phage display libraries we cloned a VHH repertoire, prepared *in vitro* from the blood leukocytes of llamas, into both a phagemid vector and a phage vector. Two VHH phage display libraries were constructed and each panned against two viral polypeptide antigens. Colony PCR and phage ELISA showed that enrichment for binders did not occur in the case of the phagemid vector-based library even after six rounds

of panning whereas high enrichments occurred in the case of the phage vector-based library at as low as round two. Following completion of pannings, for each library, four lead sdAbs were expressed and purified for subsequent binding studies by surface plasmon resonance (SPR) experiments. Consistent with the enrichment results, all four sdAbs isolated from the phagemid vector-based library did not have any binding activity whereas the ones isolated from the phage vector-based library bound to their antigen. Detailed SPR analysis on two of the four sdAb binders revealed K_Ds of interactions in the low micromolar range towards the respective antigens. These findings underline the determining role of the vector type in rescuing binders from naïve VHH phage display libraries.

2. EXPERIMENTAL

All reagents were chemical grade and were purchased from various companies. Bacterial media were prepared as described (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbour Laboratory, Cold Spring Harbour, NY (1989)). Phosphate-buffered saline (PBS) was prepared as described (Sambrook et al., supra). Oligonucleotides were purchased from Sigma Genosys (Oakville, ON, Canada). DNA sequencing was performed by the dideoxy method (Sanger et al., Biotechnology, 104-108 (1992)) using the BigDye® Terminator V1.1 Cycle Sequencing kit and ABI 3100 Genetic Analyzer (PE Applied Biosystems, Mississauga, ON, Canada). The host bacteria used for cloning was TG1: *supE hsd5 thi* (*lac-proAB*) F' [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*M15]. All the cloning steps were performed as described (Sambrook et al., supra). fd-tetGIIID phage vector was constructed from fd-tet phage vector (American Type Culture Collection, Manassas, VA) by engineering *Apa*L I and *Not* I restriction sites immediately following the gIIIp leader sequence codons (Simon J. Foote, personal communication). pJT1 phagemid vector was constructed from pSJF2 (Tanha et al., *Methods Mol. Med.*, 435-849 (2003)) by replacing c-Myc-His_5 tag with gIII downstream from the multiple cloning site. The multiple cloning site in pJT1 is modified with respect to the parent pSJF2 vector.

2.1 Construction of Llama Naïve VHH Phage Display Libraries

VHH gene repertoire was cloned as described (Tanha et al., *J. Immunol. Methods*, 97-109 (2002)) with a few modifications: cDNA was synthesized using the C_H2-specific primers, CH2FORTA4 (5'CGCCATCAAGGTACCAGTTGA3')and B3FOR (5'GGGGTACCTGTCATCCACGGACCAGCTGA3'); dsDNA was synthesized using the primers CH2FORTA4, B3FOR and FR1-specific primers VHBACKA6 (5'CGCCATCAAGGTACCAGTTGA3') and CVHHP35BACK (5'CAGGCTCAGGTACAGCTGGTGGAGTCTGG3'); the second PCR to amplify VHH genes were carried out using the FR1-specific primers 5'GATGTGCAGCTGCAGGCGTCT3' and 5'CAGGCTCAGGTACAGCTGGTG3' and the FR4-specific primer 5'TGAGGAGACGGTGACCTG3'. These primers were designed such that they either incorporated into VHH genes flanking *Apa*L I and *Not* I restriction sites for ligating into fd-tetGIIID phage vector or *BamH* I and *Bbs* I restriction sites for ligating into pJT1 phagemid vector. In the case of the phage vector, VHH phage display library construction, propagation and size determination were performed as described (Tanha et al., *J. Immunol. Methods*, 97-109 (2002)). In the case of the phagemid vector, the ligated product was desalted using spin columns and used to transform *E. coli* strain TG1. Growth of the library was performed as described (Harrison et al., *Methods Enzymol.*, 83-109 (1996)).

2.2. Panning

2.2.1 Phage vector-based library

In the instance of phage vector-based library, panning was performed as described (Tanha et al., *J. Immunol. Methods*, 97-109 (2002)) except that for the binding step phages were first pre-adsorbed in wells coated with ERNS, when the target antigen was E2, or E2 when the target antigen was ERNS and then added to the wells coated with the target antigen.

2.2.2. Phagemid vector-based library

Microtiter wells were coated overnight with 150 µl of 100 µg/ml antigen in PBS. In the morning, the wells were emptied, blotted on a paper towel and blocked with 300 µl of 2% (w/v) milk in PBS (MPBS) for 2 h at 37°C. The wells were emptied, blotted, and filled with 10^{12} pre-adsorbed phages (see 2.2.1) in 150 µl 2% MPBS. The contents were mixed by pipetting up and down and incubated at room temperature for 1.5 h. The well contents were emptied, the wells were blotted and rinsed10 times with PBS-0.1% (v/v) Tween 20 (0.1% PBST) and 10 times with PBS. The wells were emptied, blotted and the bound phages were eluted by adding 200 µl 100 mM triethylamine (35 µl triethylamine (7.18 M) in 2.5 ml water, made freshly), mixing by pipetting up and down and leaving at room temperature for 10 min. The eluted phage were transferred to a tube containing 100 µl of 1 M Tris-HCl, pH 7.4 and vortexed. 150 µl was used to infect 10 ml of exponentially growing TG1 cells (OD_{600}=0.4-0.5); the remaining was stored at -80°C. To determine the titer of the eluted phage, 100-fold serial dilutions in 500 ul 2XYT medium were made from the 10-ml culture. 100 µl of each dilution was spread on 2XYT-Amp plates and incubated at 32°C overnight. The remaining infected cells were spun down at 3300g for 10 min, re-suspended in 0.5 ml 2XYT, spread on large 2XYT-Amp plates containing 2% glucose and incubated at 32°C overnight. Further rounds of selection were performed as described (Harrison et al., *Methods in Enzymol.*, 83-109 (1996)).

2.3. Enzyme-linked Immunosorbent Assay (ELISA)

2.3.1. Phage vector-based library

Individual phage-infected TG1 colonies were used to inoculate 200 µl of LB in sterile 96-well plates. The cells were grown overnight at 100 rpm and 37°C. In the morning, the plates were spun down in a bench top centrifuge, and the sdAb phage-containing supernatant was used for phage ELISA as described below. Briefly, Nunc-Immuno MaxiSorpTM plates (Nalge Nunc International, Rochester,

NY) were coated overnight at 4°C with 150 µl of 10 µg/ml of target antigen or control proteins in PBS. The contents were removed and the plates were tapped on a paper towel to remove any liquid remaining in the wells. The wells were blocked by adding 300 µl of 2% MPBS and incubating for 2 h at 37°C. The contents of the wells were emptied as before, 100 µl of sdAb phage supernatant in 2% MPBS was added, and the wells were incubated at room temperature for 1.5 h. The wells were washed 5x with 0.05%PBST and then incubated with phage. The contents were emptied again and the wells were washed 5 times with PBST and subsequently blotted on a paper towel to remove any remaining wash buffer. 100 µl of the recommended dilution of HRP/Anti-M13 Monoclonal Conjugate (Amersham Biosciences Inc., Montreal, QC, Canada) in 2% MPBS was added and the wells were incubated at room temperature for 1 h. The wells were washed six times as before and the binding of sdAb to the antigen was detected colorimetrically by adding 100 µl of equal mixtures of TMB Peroxidase Substrate and H_2O_2 (KPL, Maryland, USA) at room temperature for several min. The reaction was stopped by adding 100 µl of 1 M H_3PO_4 and the A_{450} was measured by DYNATECH MR5000 ELISA reader (DYNATECH).

2.3.2. Phagemid vector-based library

Screening of the clones by ELISA was performed as described (Harrison et al., *Methods Enzymol.*, 83-109 (1996)).

2.4. Expression and Purification of VHHs

VHH genes were amplified from the vectors by PCR using the appropriate primers, and expressed and purified by immobilized metal affinity chromatography (IMAC) as described (Tanha et al., *Methods Mol. Med.*, 435-49 (2003)). Protein purity was determined by SDS-PAGE (Laemmeli U.K., in: Proteases and biological control [Reich et al., ed.], Cold Spring Harbour Laboratory, pp. 661-676 (1975)).

2.5. SPR Analyses

K_Ds of V_HHs were derived from SPR data collected with BIACORE 3000 biosensor system (Biacore Inc., Piscataway, NJ). 10,000 RUs of E2 and 4,500 RUs of ERNS were immobilized on research grade CM5 chips (Biacore Inc.). Ethanolamine blocked surfaces were used as references for E2 and verotoxin 1 B pentamer-coated surfaces (8,800 RUs) as references for ERNS. Immobilizations were carried out at antigen concentrations of approximately 20 µg/ml in 10mM sodium acetate buffer pH 4.5, using the amine coupling kit provided by the manufacturer. Prior to kinetic analyses, monomer V_HHs were separated using Superdex 75 10/30 HR column (Amersham Biosciences Inc.). In all instances, analyses were carried out with monomer V_HHs at 25°C in 10mM HEPES, pH 7.4 containing 150mM NaCl, 3mM EDTA and 0.005% P20 and surfaces were regenerated by washing with the running buffer. Data were fit to a 1:1 interaction model simultaneously using BIAevaluation 4.1 software (Biacore Inc.) and K_Ds were subsequently determined.

3. RESULTS

3.1. Naïve VHH Phage Display Libraries

A naïve repertoire of VHH genes were prepared from the blood leukocytes of llamas and cloned into a phage vector and a phagemid vector. The resultant vectors were used to construct two phage display libraries with diversities of 1.6×10^7 clones (phage vector-based library) and 1.8×10^8 clones (phagemid vector-based library). The utility of the two naïve libraries in yielding binders was subsequently assessed by panning against two polypeptide antigens derived from classical swine fever virus proteins E2 and ERNS (Lin et al., *J. Virol.*, 11619-11625 (2000); Langedijk et al., J. Virol. 10383-10392 (2002)). For the sake of simplicity, these antigens are referred to as E2 and ERNS.

3.2. Panning and Binding Studies

3.2.1. Phage vector-based naïve library

Two and three rounds of panning were performed against E2 and ERNS, respectively. For each antigen, 96 clones from round two were tested for binding by ELISA. For ERNS, an additional 96 clones from round three were also tested. In the case of E2, 80 of the 96 clones bound strongly to E2. The binding was specific since the positive clones did not bind to a control antigen. Sequencing of 29 randomly selected positive clones revealed three unique VHHs: E2A3, E2A2 and E2C4 (Figure 1). In the case of ERNS, 13/23 clones (round 2) and 31/32 clones (round three) bound strongly to ERNS in ELISA with no binding to a control antigen. Sequencing of 44 randomly selected ERNS binders gave three unique VHHs: ERNS-222, ERNS-216 and ERNS-E3 (Figure 1). ERNS-E3 VHH from amongst the ERNS binders and all three E2 binders were cloned into expression vector and purified to homogeneity by IMAC. All were shown to bind to their respective antigens in SPR experiments. A more detailed SPR studies on E2C4 and ERNS-E3 VHHs revealed K_Ds of interaction of 16 μM and 10 μM, respectively (Figure 2).

3.2.2. Phagemid vector-based naïve library

Four and six rounds of panning were performed against ERNS and E2 antigens, respectively. Enrichment for binders was monitored by colony PCR and phage ELISA of the eluted clones. In both cases, the percentage of the clones with VHH inserts remained very low and basically the same throughout the panning rounds, consistent with a lack of enrichment for binders. For ERNS, 14 out of 40 eluted clones had inserts with a sequence denoted as ERNS-414 (Figure 1). In the case of E2, ELISA on 96 clones from round 6 was performed. Except for 12 clones which had binding signals slightly but not significantly higher than the background, the remainder did not bind to the target antigen. Sequencing of these 12 clones gave three unique VHH sequences: E2-1, E2-2 and E2-S (Figure 1). E2-2 and E2-S were aberrant: E2-2 had a deletion of 7 residues in its FR1 and E2-S had an amber stop codon. All three VHHs as well as the above-mentioned ERNS-414 VHH were expressed and purified by IMAC for subsequent

SPR binding studies. In the case of E2-S, the amber stop codon was replaced with an appropriate codon to generate a complete reading frame. All four VHHs showed no binding to their respective antigens In SPR experiments.

Figure 1. CDR sequences of VHHs isolated by panning the phage vector-based (A) and the phagemid vector-based (B) display libraries against ERNS and E2 antigens. VHH signature residues at position 37, 44, 45 and 47 are also included.

(A) Antigen: ERNS

VHH	37	44	45	47	CDR1	CDR2	CDR3
ERNS-222	Y	Q	R	L	INAMG	TIHSGGSTNYADSVKG	LIRNSDYDYDPEYAMNY
ERNS-216	Y	Q	R	L	IQAMG	RIGSGGITDYADSVKG	ATYDRVAGWSNF
ERNS-E3	F	E	R	F	NYAMG	AITTSGATTSVADSVKG	ARGYDRY

(A) Antigen: E2

VHH	37	44	45	47	CDR1	CDR2	CDR3
E2A3	F	E	R	F	RYNMG	AFRWITGTTQYANSAKG	DPRVTFNVNEFDY
E2A2	Y	Q	R	L	INDMG	VITSDYSTNYADSVKG	QLTSGFSSLSGSYTRTGDYNY
E2C4	F	D	R	F	TYAMA	RINWRSSSIYYADSVKG	DTLGPDYSNY

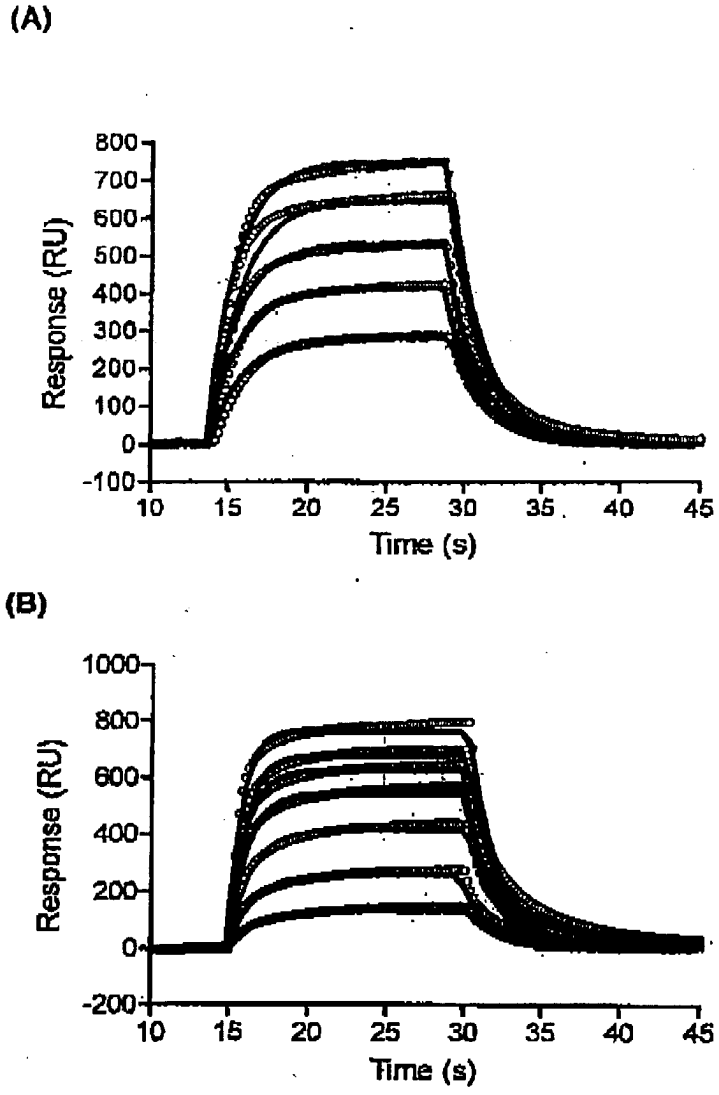
(B) Antigen: ERNS

VHH	37	44	45	47	CDR1	CDR2	CDR3
ERNS-414	F	E	R	F	SYAMA	TVSENGNAFYADSVKG	GRLSASMRDYDQ

(B) Antigen: E2

VHH	37	44	45	47	CDR1	CDR2	CDR3
E2-1	F	Q	R	H	MYVWG	HITGGGTTIYADSAES	GETTWHLGKLGFGS
E2-2	F	E	R	F	SYVMG	ISWSGSSTYYADSVKG	GPPLARTPPQRPVSYRY
E2-5	F	E	R	P	NYGMG	QINYSGDTTVYADSVKG	VRTYYSGSDLLSGHRYNY

Figure 2. Sensorgram overlays showing the binding of 1.1, 1.7, 2.3, 2.8 and 3.4 μM ERNS-E3 VHH to immobilized ERNS (A), 2, 4, 8, 12, 16, 20 and 26 μM E2C4 VHH to immobilized E2 (B), and the global fitting of the binding data to a 1:1 interaction model. The open circles represent the data points and solid lines the best fit to the model.



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Jan 12, 2005 — Date

J. Tanha — Jamshid Tanha

Witness Signature C. Roger MacKenzie

Witness Name Printed C. Roger MacKenzie Date January 12, 2005.